The envelope membrane of rat liver nuclei contains a P-type Ca\textsuperscript{2+}-transporting pump, revealed by the presence of a Ca\textsuperscript{2+}-stimulated phosphoenzyme. The level of the nuclear phosphoenzyme in autoradiographed polyacrylamide gels was decreased by lanthanum, as typically observed in the endoplasmic reticulum Ca\textsuperscript{2+} pump. It was also decreased by thapsigargin and 2,5-di-(tert-butyl)-1,4-benzohydroquinone, two accepted inhibitors of the endoplasmic reticulum Ca\textsuperscript{2+}-ATPase. Comparative proteolysis of the phosphorylated enzyme of liver microsomes (endoplasmic reticulum) and nuclear membranes revealed an identical cleavage pattern. In addition, antibodies raised against the endoplasmic reticulum Ca\textsuperscript{2+} pump cross-reacted with the pump in the nuclear membranes. The findings show that nuclear membranes contain a Ca\textsuperscript{2+}-transporting pump closely related to that of the endoplasmic reticulum. The pump is likely to be involved in the control of nuclear free calcium.

Ca\textsuperscript{2+} is involved in the regulation of fundamental nuclear processes. The onset of DNA replication is very sensitive to changes in the level of Ca\textsuperscript{2+} (Whitfield et al., 1976, 1987; Whitfield, 1990; Hazeltin et al., 1979). In line with accepted ideas on the role of calmodulin in cytoplasmic processes, it has been suggested that calmodulin is a mediator of the Ca\textsuperscript{2+} signal also in the nucleus. In fact, prior to DNA synthesis, the level of nuclear calmodulin significantly increases (Chafouleas et al., 1981, 1982; Serratosa et al., 1988) and calmodulin becomes specifically associated with a Ca\textsuperscript{2+}-dependent way with the nuclear matrix; the latter is the site of DNA replication (Serratosa et al., 1988). In addition, Ca\textsuperscript{2+}, in conjunction with calmodulin and/or other factors, is involved in the control of the expression of some genes (White, 1985; White and Bankroft, 1987), of DNA metabolism (Chafouleas and Meana, 1984; Chafouleas et al., 1984; Charp and Regan, 1985), and in the maintenance of the structure of the nucleus (Bachs et al., 1990). All these findings demand that the nuclear Ca\textsuperscript{2+} content be strictly controlled. The suggestion that nuclear Ca\textsuperscript{2+} is not in free equilibrium with the cytoplasm is not obvious. The nucleus is surrounded by a double membrane, the nuclear envelope, the inner leaflet of which defines the nucleus itself and is in contact with the nuclear lamina, a meshwork of intermediate filament-like proteins. The outer nuclear membrane of most cells is continuous with the endoplasmic reticulum with the result that the space between the outer and inner membranes, the perinuclear space, is continuous with the lumen of endoplasmic reticulum. The inner and outer nuclear envelope membranes join periodically at the nuclear pore complexes, which are large supramolecular structures that form channels for the exchange of materials between the nucleus and the cytoplasm. The nuclear pores are not an obligatory barrier for ions or small macromolecules, which freely diffuse through them (Paine et al., 1975; Lang et al., 1986).

Based on these structural features, the nucleus has long been thought to freely exchange Ca\textsuperscript{2+} with the cytoplasm. However, measurements of intracellular Ca\textsuperscript{2+} in single cells with fluorescent indicators in conjunction with imaging microscopy have shown that Ca\textsuperscript{2+} gradients do exist between the cytosol and the nucleus. They have also shown that the nucleus does not undergo the same changes in free Ca\textsuperscript{2+} in response to stimuli as the surrounding cytoplasm. Williams et al. (1987), using Fura-2 in smooth muscle cells stimulated by depolarization, have reported that the cytoplasmic Ca\textsuperscript{2+} rises above 800 nM during stimulation, whereas intranuclear Ca\textsuperscript{2+} only rises from 200 nM at rest to no more than 300 nM. Similar results have been obtained on smooth muscle cells stimulated by agonists (Neylon et al., 1990). Confocal microscopy, due to the better resolution of signals and cellular substructures, has contributed to the precise localization of Ca\textsuperscript{2+} transients in single cells. Hernandez-Cruz et al. (1990), using Fluo-3 in voltage-clamped neurons, have also seen that nuclear Ca\textsuperscript{2+} is independently regulated but in the opposite way: after electrical stimulation signals in the nucleus were larger and decayed more slowly than in the cytoplasm. Waybill et al. (1990) have studied the effects of vasopressin and the epidermal growth factor on nuclear Ca\textsuperscript{2+} in cultured rat hepatocytes; in resting hepatocytes cytosolic Ca\textsuperscript{2+} was about 2-fold higher than in the nucleus and increased upon stimulation in both compartments without changes in the gradient between the two pools. Thus, mechanisms evidently exist to screen the nucleus from cytoplasmic Ca\textsuperscript{2+} transients. Recently, two Ca\textsuperscript{2+}-transporting mechanisms have been described in isolated rat liver nuclei: an ATP-dependent uptake and an Ins\textsubscript{P\textsubscript{3}}-induced\textsuperscript{1} release. Isolated nuclei incubated in the presence of ATP and submicromolar Ca\textsuperscript{2+} accumulated large amounts of it; part of the accumulated Ca\textsuperscript{2+} was released by Ins\textsubscript{P\textsubscript{3}} (Nicotera et al., 1990; Malviya et al., 1990). Ins\textsubscript{P\textsubscript{3}} receptors in nuclear membranes have also been detected by

\textsuperscript{1}The abbreviations used are: Ins\textsubscript{P\textsubscript{3}}, 1,4,5-inositol trisphosphate; PMSF, phenylmethylsulfonyl fluoride; DTT, 1,4-dithiothreitol; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitriilo)tetraacetic acid; PBS, phosphate-buffered saline.
Nuclear Calcium Pump

In the present study, the ATP-dependent Ca\(^{2+}\) uptake activity of isolated rat liver nuclei has been characterized. A Ca\(^{2+}\) pump probably identical to that of the endoplasmic reticulum has been identified. This pump is likely to be one of the mechanisms of the nuclear envelope responsible for the regulation of nuclear Ca\(^{2+}\). A model for the control of nuclear Ca\(^{2+}\) is proposed in which the perinuclear space plays the role of a dynamic Ca\(^{2+}\) pool.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents used were analytical grade. Bovine pancreas deoxyribonuclease (I DNase I), ribonuclease A (RNase A), and bovine pancreas \(\alpha\)-chymotrypsinogen A were from Sigma. (\(\gamma-\text{P}\)) ATP (3 Ci/mmol) and 45 CaCl\(_2\) (2 mCi/ml) were from American Sham International (Amsterdam, The Netherlands). Nitrocellulose membranes for immunoblots were from Schleicher and Schuell (Dass-,

Preparation of Nuclei—Rat liver nuclei were isolated essentially according to Nicotera et al. (1989). All steps of the preparation were performed at 4 °C. The removed livers were immediately perfused with about 50 ml of ice-cold 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 22,000 g centrifugation was centrifuged at 49,000 g.

**Isolation of Nuclear Envelopes—**Nuclear envelopes were isolated as described by Dwyer and Blobel (1976). All solutions contained 0.5 mM PMSF and 1 mM DTT. The tubes were gently mixed, and a 6-ml cushion of TKM containing 2.5 mg/ml of RNase A (2.5 mg/ml in H\(_2\)O) and 2.5 μl of RNase A (2.5 mg/ml in H\(_2\)O) were added. After incubation for 15 min at room temperature the suspension was distributed between two SW 28 tubes, and each aliquot was underlaid with 12.5 ml of STM buffer and centrifuged as described above. The pellets (nuclear envelopes) were resuspended with 0.1 M EGTA and 20 μl of 0.1 M CaCl\(_2\) containing traces of Ca\(^{45}\) (45 μCi/ml 0.1 M CaCl\(_2\)) were added to 1 ml of nuclear suspension obtained as described above so that the final free Ca\(^{2+}\) concentration amounted to 400 nM according to Bertay (1978). The nuclei were incubated for 2 min at 37 °C, and ATP was added to a final concentration of 1 mM. 50-100-μl aliquots were then quickly filtered at different times through nitrocellulose filters (2.5-cm diameter, 0.45-μm pore size), previously incubated in incubation buffer containing 2 mM EGTA. The filters were washed twice with 1 ml of incubation buffer and their radioactivity counted with a liquid scintillation counter. Ruthenium red, the calcium ionophore A23187, and other effectors were added before starting the uptake reaction and incubated with the nuclei for 2 min at 37 °C.

**Calcium Uptake Measurements**—Calcium uptake by isolated rat liver nuclei was measured as described by Nicotera et al. (1989). 10 μl of 0.1 M EGTA and 20 μl of 0.1 M CaCl\(_2\) containing traces of Ca\(^{45}\) (45 μCi/ml 0.1 M CaCl\(_2\)) were added to 1 ml of nuclear suspension obtained as described above so that the final free Ca\(^{2+}\) concentration amounted to 400 nM according to Bertay (1978). The nuclei were incubated for 2 min at 37 °C, and ATP was added to a final concentration of 1 mM. 50-100-μl aliquots were then quickly filtered at different times through nitrocellulose filters (2.5-cm diameter, 0.45-μm pore size), previously incubated in incubation buffer containing 2 mM EGTA. The filters were washed twice with 1 ml of incubation buffer and their radioactivity counted with a liquid scintillation counter. Ruthenium red, the calcium ionophore A23187, and other effectors were added before starting the uptake reaction and incubated with the nuclei for 2 min at 37 °C.

**Identification of the Phosphoenzymes**—Intact nuclei, nuclear envelopes, and crude microsomes were suspended in 100 mM KCl, 2 mM Tris, pH 7.0, and incubated for 10 s at 25 °C with 30 μl of (\(\gamma-\text{P}\)) ATP (3 mM final ATP concentration). The reaction was carried out in the presence of either 2 mM EGTA or 50 μM CaCl\(_2\) or 50 μM LaCl\(_3\). The inhibitors of the endoplasmic reticulum, Ca\(^{2+}\)-ATPase, 2,5-di-(tert-butyl)-1,4-benzhydroquinone, and ruthenium red were incubated with the membranes in the presence of 50 μM CaCl\(_2\) for 5 min at 37 °C prior to performing the phosphorylation reaction. The reaction was stopped by the addition of 0.6 ml of 8% trichloroacetic acid. The precipitated protein was pelleted and the pellet washed once with H\(_2\)O. The labeled proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Amory et al., 1975) and detected by autoradiography of the dried gels on x-ray Kodak films after 2-12 h of exposure at −70 °C.

**Digestion of the Phosphoenzymes**—The protein pellet obtained after treatment of nuclear and microsomal membranes with (\(\gamma-\text{P}\)) ATP as described above was suspended in 50 μl of sample buffer for acidic gels (Amory et al., 1975). Chymotrypsin or other proteases were added (0.1 μg/μg of total protein), and the mixture was incubated for 20 min at room temperature. After incubation the digested samples were loaded on 15% acidic gels. The phosphorylated fragments were visualized by autoradiography as described above.

**ImmunobLOTS**—Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (1977) were transferred onto nitrocellulose according to Towbin et al. (1979) for 2 h at 300 mA using a Bio-Rad Mini Trans-Blot apparatus. After the transfer, the filters were saturated in PBS buffer (0.14 M NaCl, 10 μM phosphate buffer, pH 7.5) containing 4% milk powder (incubation buffer) or 1% milk powder (incubation buffer) for 1 h at room temperature or overnight at 4 °C. The immunoresponse was performed in PBS buffer containing 2% milk powder (incubation buffer) for 1 h at room temperature or overnight at 4 °C. The antibodies were used in dilutions ranging from 1:100 to 1:500. After washing, the antigen-antibody interaction was visualized with a horseradish peroxidase-linked secondary antibody diluted 1:1000 in incubation buffer for 1 h at room temperature. The blots were stained with 0.5 mg/ml 4-chloro-1-naphthol (stock solution: 3 mg/ml in methanol) and 0.015% H\(_2\)O\(_2\) in PBS buffer after washing with PBS buffer.

**Protein Determination**—The protein concentration was determined as described by Lowry et al. (1951) with bovine serum albumin for calibration.

**Marker Enzymes**—The activity of cytochrome oxidase (mitochondrial marker) was determined using a Clark oxygen electrode as described by Wharton and Tsagoloff (1967). The activity of 5'-nucleotidase (plasma membrane marker) and glucose-6-phosphatase (endoplasmic reticulum marker) were measured according to Aronson and Touster (1979) and Swanson (1955), respectively.

**RESULTS**

**Isolation of Nuclei and Calcium Uptake Measurements**—The method used for the isolation of rat liver nuclei capable of accumulating calcium in an ATP-dependent way (Nicotera et al., 1989) yielded a fraction highly enriched in intact nuclei. Analysis of marker enzymes showed a low degree of contamination by other structures. The total activities of cytochrome...
Nuclear Calcium Pump

oxides in the presence of the calcium ionophore A23187 (5 pg/ml) for

The Calcium-dependent Phosphoenzyme in the Nuclear
Membranes—To identify the protein responsible for the ATP-
dependent uptake of Ca\(^{2+}\) into isolated nuclei, the formation of a Ca\(^{2+}\)-dependent phosphorylated intermediate was inves-
tigated. This is based on the plausible assumption that nuclear
membranes might possess a Ca\(^{2+}\)-transporting pump belong-
ing to the P-type (Pedersen and Carafoli, 1987a, 1987b), which
forms an aspartyl phosphate during the catalytic cycle. Fig. 2
shows that nuclear envelope fractions incubated with Ca\(^{2+}\)
and γ-labeled ATP indeed contained a phosphoenzyme of
molecular mass of about 110 kDa. The phosphoenzyme was
Ca\(^{2+}\)-dependent and inhibited by La\(^{3+}\). The latter finding is
typical of the reticular type Ca\(^{2+}\) pump and of all other P-
type pumps with the exception of the plasma membrane Ca\(^{2+}\)-
ATPase (Schatzmann and Buergin, 1978; Szasz et al., 1978;
Wuytack et al., 1987). This indicated that the Ca\(^{2+}\)-ATPase
present in the nuclear membranes was analogous to that of
endoplasmic reticulum and different from that of the plasma
membrane. In fact, a comparison with a preparation of liver
endoplasmic reticulum showed that the gel migration of the
Ca\(^{2+}\)-dependent phosphorylated intermediate of the latter was
identical to that of the nuclear fractions (Fig. 2).

A Comparison of the Phosphoenzymes of the Nuclear
Membranes and the Endoplasmic Reticulum—To explore the sim-
ilarity between the endoplasmic reticulum and the nuclear
Ca\(^{2+}\)-ATPase in more detail, the phosphoenzymes formed in
the two membrane systems were digested with proteases, and
the fragmentation patterns of the labeled fragments were
compared. Fig. 3 shows that both the endoplasmic reticulum
and the nuclear envelopes, after digestion with chymotrypsin,
formed a 35-kDa Ca\(^{2+}\)-dependent phosphorylated fragment.
(The phosphorylated band of 25 kDa present in the endo-
plasmic reticulum preparation probably arose from a protein
distinct from the Ca\(^{2+}\)-ATPase, since its phosphorylation was
Ca\(^{2+}\)-independent.) The effect of specific inhibitors of the
endoplasmic reticulum pump on the formation of the phos-
phoenzyme was also studied. Fig. 4 shows that 10 μM 2,5-di-
(tart-butyl)-1,4-benzohydroquinone or 1 μM thapsigargin,
both accepted inhibitors of the reticular pump (Moore et al.,
1987; Takemura et al., 1989), strongly decreased the formation
of the Ca\(^{2+}\)-dependent phosphoenzyme in both endoplasmic
reticulum and nuclear envelopes. The inhibition was essen-
tially complete in the former and very marked, although not
complete, in the latter. The difference could be due to exper-
imental aspects, e.g. different protein concentration, in the
two systems. This indicated that the nuclear membranes
contained a Ca\(^{2+}\)-ATPase closely related or identical to that of
the endoplasmic reticulum.

Immunological Analysis of the Ca\(^{2+}\)-ATPases in the Endo-
plasmic Reticulum and Nuclear Membranes—After gel sepa-
ration and blotting, nuclear membranes reacted with antibod-
ies to the endoplasmic reticulum pump (Fig. 5), confirming that
the Ca\(^{2+}\)-ATPase present in the nuclear membranes was
closely related to that of endoplasmic reticulum. The band of
170 kDa seen in the endoplasmic reticulum blot probably
became stained because of the non-absolute specificity of the

![Fig. 1. ATP-dependent Ca\(^{2+}\) uptake by isolated rat liver nuclei. Isolated nuclei (closed circles) were incubated at 37 °C with 1 mM ATP in the presence of 400 nM free calcium and trace amounts of "Ca" and filtered at the times indicated (closed circles). As a control (open circles), nuclei were incubated under the same conditions in the presence of the calcium ionophore A23187 (5 μg/ml) for 2 min at 37 °C prior to the addition of ATP (open circles). For additional details see "Experimental Procedures."](image1)

![Fig. 2. Identification of the Ca\(^{2+}\)-dependent phosphoenzymes. The figure shows the autoradiography of the acidic gels (8%) with the phosphorylated proteins. NE and M stand for nuclear envelopes and microsomes (endoplasmic reticulum). Lane 1, 2 mM EGTA; lane 2, 50 μM CaCl\(_2\); lane 3, 50 μM CaCl\(_2\) and 50 μM LaCl\(_3\). The numbers on the left are the masses (kDa) of the standard proteins. The experimental conditions are described under "Experimental Procedures."](image2)

![Fig. 3. Comparative proteolysis of the phosphoenzymes. Autoradiography of the acidic gel (15%) of the fragments obtained after incubation of the phosphoenzyme of nuclear envelopes (NE) and microsomes (M) with chymotrypsin (0.1 mg/mg protein) for 20 min at room temperature. Lane 1, 2 mM EGTA; lane 2, 50 μM CaCl\(_2\). The experimental conditions including the phosphoenzyme formation are described under "Experimental Procedures." The arrowheads point to the fragments phosphorylated in a Ca\(^{2+}\)-dependent way. The numbers on the left are the masses (kDa) of the standard proteins.](image3)
DISCUSSION

The work by Nicotera et al. (1989) has clearly shown that the nucleus contains an ATP-dependent Ca\(^{2+}\) transport mechanism with a relatively high affinity for Ca\(^{2+}\). Analysis of the phosphoenzyme typical of P-type-transporting ATPases has shown that the nuclear pump whose molecular mass was shown to be 2,5-di-(tert-butyl)-1,4-benzohydroquinone, while inhibiting the endoplasmic reticulum Ca\(^{2+}\)-ATPase (Moore et al., 1987) had no effect on the nuclear ATP-dependent uptake system. However, in the present work, this inhibitor, as well as thapsigargin (another accepted inhibitor of the endoplasmic reticulum Ca\(^{2+}\)-ATPase (Takemura et al., 1989)), affected the phosphoenzymes of both pumps. This would indicate that the ATP-dependent Ca\(^{2+}\) uptake of isolated nuclei studied by Nicotera et al. (1989) is catalyzed by a system distinct from the pump identified in the nuclear envelope in the present study and possibly different from P-type cation-transporting ATPases.

Very likely, Ca\(^{2+}\)-ATPase molecules are present on both the outer and inner nuclear membranes. A number of recent contributions, using fluorescent indicators in conjunction with imaging microscopy, have established that gradients of free Ca\(^{2+}\) exist between the cytoplasm and the nucleus. Nuclear free calcium is higher than that in the cytoplasm in some cells (Williams et al., 1985, 1987; Neylon et al., 1990), but the situation is apparently the opposite in other cells, e.g., the hepatocyte (Chandra et al., 1989; Hernandez-Cruz et al., 1990; Waybill et al., 1990). Active transport mechanisms, e.g., pumps, must thus exist to produce and maintain the imbalance. The findings in the present study are best interpreted by suggesting that the space between the two nuclear membranes plays a major role in the regulation of the Ca\(^{2+}\) content in the nucleus. Due to the continuity between the nuclear outer membrane and the endoplasmic reticulum, the perinuclear space could be considered equivalent to the cisternae of the endoplasmic reticulum in terms of Ca\(^{2+}\) transport and of the InsP\(_3\) receptors; the latter have also been identified in the nuclear membrane (Ross et al., 1989; Nicotera et al., 1990; Malviya et al., 1990). Thus, Ca\(^{2+}\) would be accumulated by the pump in the space between the two membranes, and InsP\(_3\) would interact with the membrane to discharge it mostly from the cytoplasmic side, based on the assumption that InsP\(_3\) is present essentially there. InsP\(_3\) would thus release a transient of calcium at the nuclear periphery in response to first messenger challenges to the cell. The Ca\(^{2+}\) pool in the perinuclear space would thus be involved in the production of local Ca\(^{2+}\) signals, at the same time protecting the nucleus from unwanted Ca\(^{2+}\) increases. This proposal agrees with the observations made by Pujol et al. (1989) on the Ca\(^{2+}\)-dependent association of calmodulin with the nuclear matrix during the proliferation of hepatocytes (Serratosa et al., 1988); the injection of \(\alpha\)-adrenergic blockers into hepatotomized rats inhibited the redistribution of nuclear membrane and calmodulin. The \(\alpha\)-agonist adrenalin, which releases Ca\(^{2+}\) from endoplasmic reticulum, removed the inhibition. Endoplasmic reticulum-like Ca\(^{2+}\) stores are thus involved in the regulation of the association of calmodulin with the nuclear matrix.

Ca\(^{2+}\) imaging results in intact cells have shown that Ca\(^{2+}\) changes occur at the nuclear periphery (Williams et al., 1987; Chandra et al., 1989; Hernandez-Cruz et al., 1990); they could, however, arise from endoplasmic reticulum vesicles rather than from the nuclear envelope itself. Using monoclonal antibodies directed to the Ca\(^{2+}\)-ATPase of skeletal muscle sarcoplasmic reticulum, Burgoyne et al. (1989) detected two Ca\(^{2+}\)-ATPase-like proteins in chromaffin cells: a 100-kDa protein diffusely distributed in the cell and a 140-kDa protein restricted to a region closely surrounding the nucleus. They indicated that the effects of InsP\(_3\) were initiated in a region close to the nucleus. The authors suggested two types of endoplasmic reticulum Ca\(^{2+}\) stores and proposed that the store sensitive to InsP\(_3\) plays a major role in the regulation of Ca\(^{2+}\)-dependent events within the nucleus.

An ATP-dependent transport system located in the nuclear
pore complex has also been suggested (Nicotera et al., 1990), leading to the proposal that the nuclear Ca²⁺ store could be involved in the regulation of cytosolic Ca²⁺. The release of Ca²⁺ from the nucleus to the cytoplasm through the InsP₃ receptor channel would be the second component in the process. The proposal is not easy to reconcile with the findings (see above) that in some cells (e.g. hepatocytes) Ca²⁺ inside the nucleus is apparently lower than in the cytoplasm, the gradient being maintained after hormonal stimulation (Chandra et al., 1989; Hernandez-Cruz et al., 1990; Waybill et al., 1990); in these cells the ATP-driven pumping mechanism would transport Ca²⁺ downhill, from the cytosol into the nucleus.

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REFERENCES

Hernandez-Cruz, A., Sala, F., and Adams, P. R. (1990) Science 247, 858-862
Swanson, M. A. (1965) Methods Enzymol. 2, 541-543